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Oligonucleotide Primer, and siRNA Analysis on a ProSwift High Resolution Weak Anion-Exchange Monolith

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ABSTRACT

Anion-exchange chromatography is effective for oligonucleotide separations. This technique is used for evaluation of synthetic oligonucleotide coupling efficiency, purity assessment, and verification of the components in nucleic acid amplification mixtures. The ProSwift™ WAX-1S is a new monolithic anion-exchange column that we demonstrate is useful for oligonucleotide assays. The monolith consists of a single cylindrical polymer rod containing an interconnected, uninterrupted matrix of pores and channels. Due to the monolithic structure, diffusive mass transfer is minimal, and convective mass transfer dominates, resulting in high efficiency separations. This is especially true for larger molecules with low diffusivities like oligonucleotides and proteins.

Many new applications for RNA interference are based on relatively short oligomers (21–24 bases). The diversity of RNAi applications encompasses: possible anti-malarial drugs, RNA-virus infection treatments, transcriptional gene silencing, control of RNA splicing isoforms, and many others. While RNA can be produced by different synthetic routes, all of the syntheses currently employed may produce occasional aberrant 2´– 5´ linkages. The presence of these linkage isomers has been shown to have biochemical effects, but an assay for the presence of those linkage isoforms has not been described. Since the 3´– 5´ linkage and the 2´– 5´ linkage do not result in products with different masses, simple MS analyses will not differentiate between RNA with, and without, these aberrant linkages.

We will show that the ProSwift WAX and the DNAPac® pellicular anion exchangers can resolve many of these linkage isomers. We will also show anion exchange separations that provide confirming evidence for the presence of 2´– 5´ linkages in RNA, after partial degradation.

We will also illustrate the use of the ProSwift WAX-1S monolith for separation of oligonucleotides having different fluorescent probes (as RT- and Q-PCR reporters) and by oligonucleotide length.

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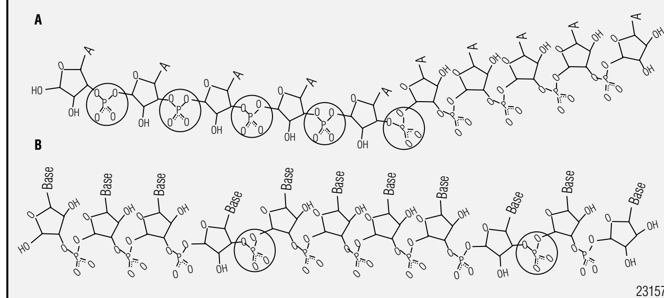
OLIGONUCLEOTIDE LINKAGE VS RETENTION ON HIGH RESOLUTION ANION-EXCHANGE COLUMNS

RNA synthetic routes may produce occasional 2´– 5´ phosphodiester bonds where biologically normal RNA has 3´– 5´ linkages. At the TIDES 2005 conference, the presence of these aberrant linkages was considered to have biological consequences, and presented a difficult analytical challenge because:

1. They do not introduce a change in mass that might be detected by mass spectrometry.
2. They are unlikely to alter hydrophobicity that would allow resolution by reversed-phase chromatography.
3. They are unlikely to alter ionic character in RNA that would allow resolution by ion-exchange chromatography.

Figure 1. Ribonucleic Acid (RNA) Linkage Isomer Placement



In Figure 1A, rA oligomers with 2´– 5´ linkages at their 5´ ends were produced at Ambion to evaluate the ability of DNAPac and ProSwift columns to differentiate them from normal 21-mers. Mixed-base RNAs with linkage isoforms at selected positions in the 21 mers produced at Ambion (Figure 1B) were used to evaluate anion-exchange resolution of RNA with only one or two aberrant 2´– 5´ linkages placed at non-adjacent positions.

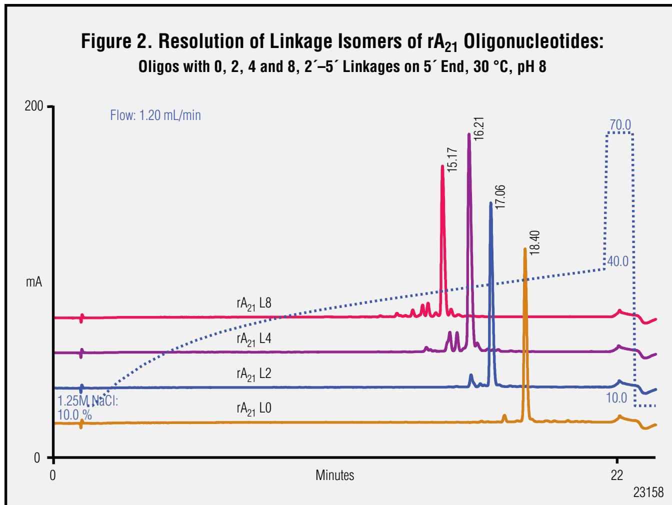


Figure 2. Resolution of Linkage Isomers of rA_{21} Oligonucleotides: Oligos with 0, 2, 4 and 8, 2´–5´ Linkages on 5´ End, 30 °C, pH 8

In Figure 2, a curved NaCl gradient applied to the DNAPac PA200 column readily resolves the crude rA_{21} mer RNAs in order of decreasing number of 2´– 5´ linkages.

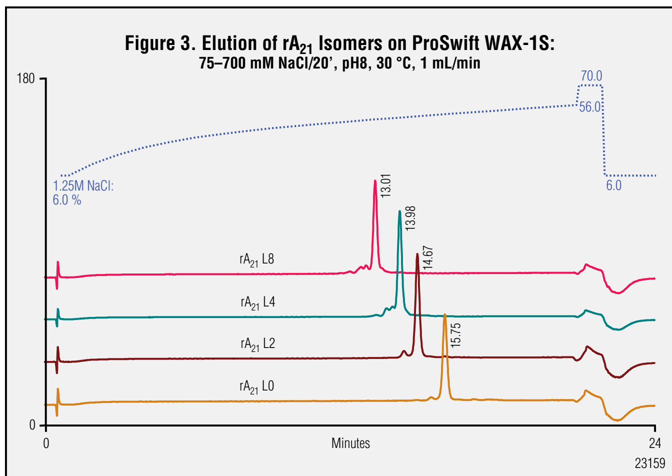


Figure 3. Elution of rA_{21} Isomers on ProSwift WAX-1S: 75–700 mM NaCl/20', pH8, 30 °C, 1 mL/min

Similarly, a curved NaCl gradient applied to the ProSwift WAX-1S column readily resolves the crude rA_{21} mer RNAs, also in order of decreasing number of 2´– 5´ linkages (Figure 3). RNA isoforms with inadvertently-generated aberrant linkages will not likely have such multiple linkages or have them adjacent to one another, and will have a mixed-base composition.

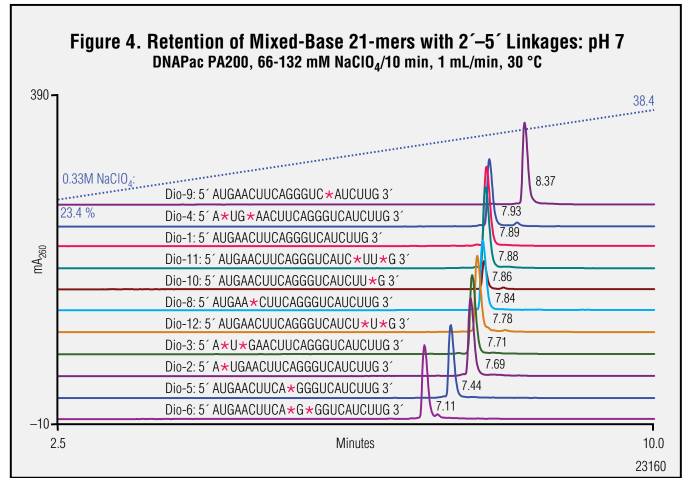


Figure 4. Retention of Mixed-Base 21-mers with 2´–5´ Linkages: pH 7 DNAPac PA200, 66-132 mM $NaClO_4$/10 min, 1 mL/min, 30 °C

In Figure 4, a suite of mixed-base 21-mers with aberrant linkages were chromatographed on the DNAPac PA200 at pH 7. At this pH samples Dio-5, Dio-6, and Dio-9 are completely resolved from all other samples. Note that some aberrant linkage containing oligos elute before, and some elute after the RNA containing only normal linkages.

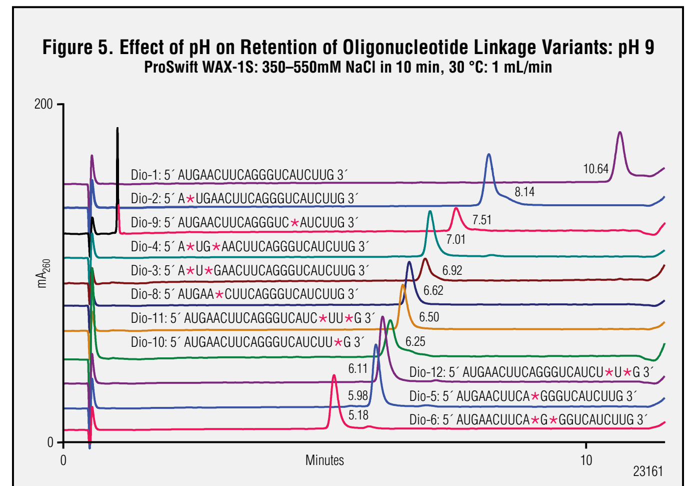


Figure 5. Effect of pH on Retention of Oligonucleotide Linkage Variants: pH 9 ProSwift WAX-1S: 350–550mM NaCl in 10 min, 30 °C: 1 mL/min

In Figure 5, the same samples are chromatographed on the ProSwift WAX-1S. All components are at least partially resolved. At pH 9, all of the RNAs harboring aberrant linkages elute significantly ***earlier*** than the 21-mer having only normal 3´– 5´ linkages. Since they elute earlier, they are not readily differentiated from failure sequences. Hence an independent confirming method is needed to verify their linkage status.

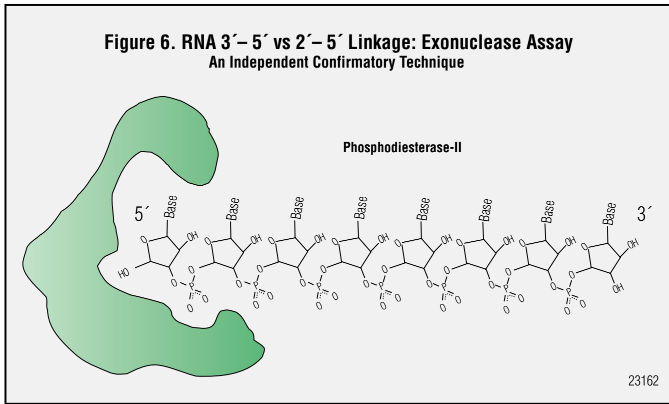


Figure 6. RNA 3´– 5´ vs 2´– 5´ Linkage: Exonuclease Assay
An Independent Confirmatory Technique

Phosphodiesterase-II excises terminal monophosphates from the 5´ end of oligonucleotides, producing 3´ monophosphates (Figure 6). If this exonuclease fails to process through 2´– 5´ linkages, it should produce oligonucleotide fragments indicating the presence of those aberrant linkages.

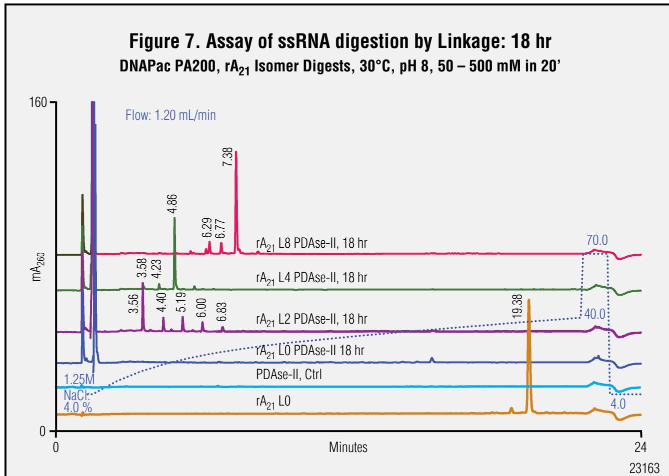


Figure 7. Assay of ssRNA digestion by Linkage: 18 hr
DNAPac PA200, rA_{21} Isomer Digests, 30°C, pH 8, 50 – 500 mM in 20'

Undigested rA_{21} mers with no aberrant linkages elute at 19.38 minutes, and exposure of this oligo to PDAse-II results in complete digestion (Figure 7). Exposure of this compound with 2, 4, or 8 aberrant linkages to PDAse-II for 18 hours reveals major digestion products at ~ 3.56 (rA_{21}L2), 4.86 (rA_{21}L4), and 7.38 (rA_{21}L8) minutes. The DNAPac PA200 elution position of each of these fully digested products is consistent with oligos of length equal to the number of consecutive aberrant linkages.

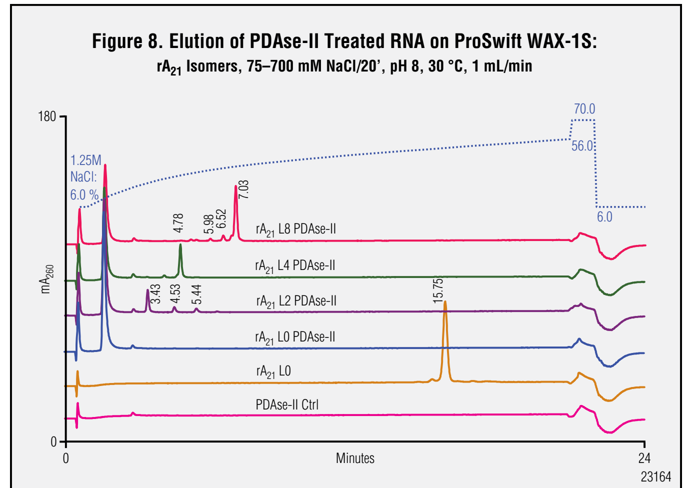


Figure 8. Elution of PDAse-II Treated RNA on ProSwift WAX-1S:
rA_{21} Isomers, 75–700 mM NaCl/20', pH 8, 30 °C, 1 mL/min

A repeat of the rA_{21} mer DNAPac study on the ProSwift WAX monolith (Figure 8) produced the same result. The undigested oligo elutes at 15.75 min, and the elution positions of the digestion products are consistent with the number of consecutive aberrant linkages. These results also demonstrate that Phosphodiesterase-II can *skip over* the 5' end located aberrant linkages and perform endonucleolytic cleavage of the normal linkages before continuing its normal exonuclease activity.

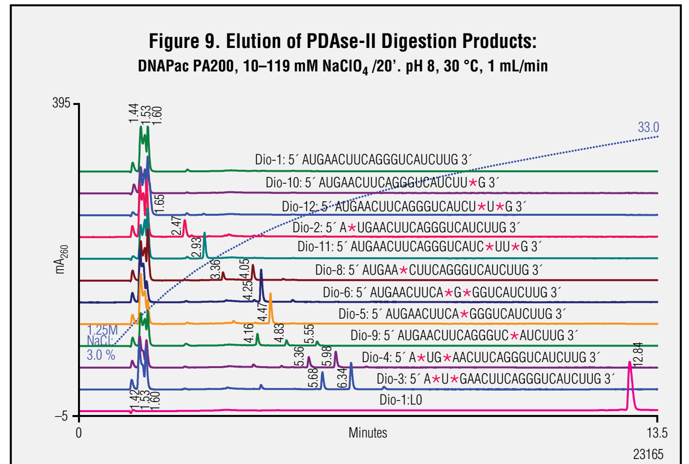


Figure 9. Elution of PDAse-II Digestion Products:
DNAPac PA200, 10–119 mM $NaClO_4$ /20'. pH 8, 30 °C, 1 mL/min

DNAPac PA200 analysis of PDAse-II treated mixed-base RNA 21-mers (having only one or two aberrant 2´– 5´ linkages at different positions in the RNA) result in specific, differentially retained, degradation products (Figure 9). The chromatographic elution position appears to be indicative of the length of the digested fragment.

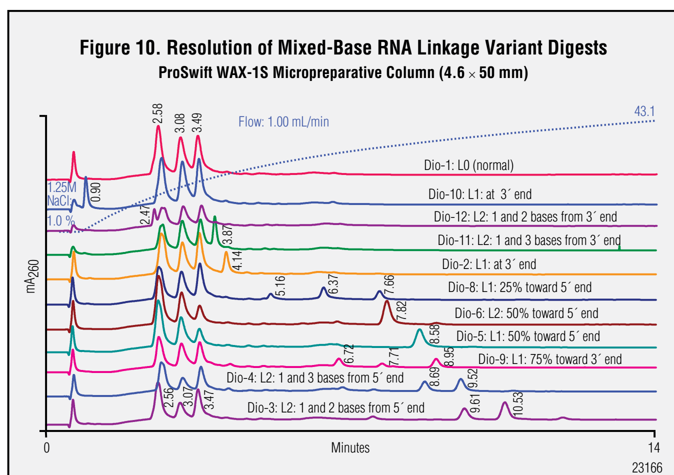


Figure 10. Resolution of Mixed-Base RNA Linkage Variant Digests
ProSwift WAX-1S Micropreparative Column (4.6 × 50 mm)

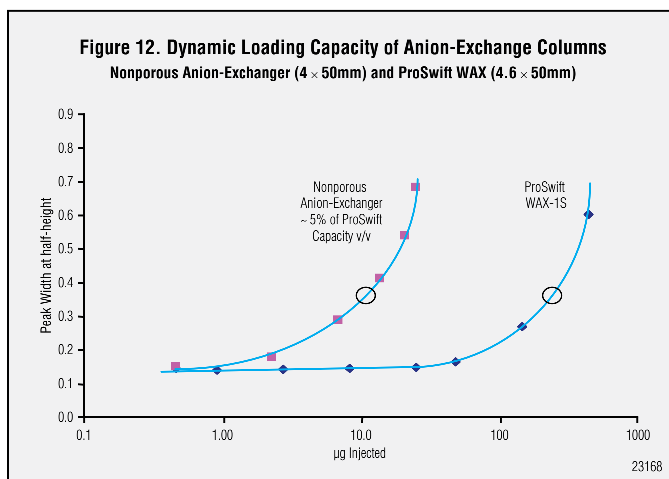


Figure 12. Dynamic Loading Capacity of Anion-Exchange Columns
Nonporous Anion-Exchanger (4 × 50mm) and ProSwift WAX (4.6 × 50mm)

ProSwift WAX monolith analyses of PDAse-II treated mixed-base RNA 21-mers containing one or two aberrant 2´– 5´ linkages at different positions in the RNA, also result in specific, differentially retained, degradation products. Elution time again appears to reflect the length of the digested fragments even at times preceding elution of the mono-phosphates (2.5 – 3.5 min) (Figure 10).

In Figure 12, comparing the peak width (at half-height) of samples injected onto ProSwift monolith and nonporous anion-exchange columns, we observe a significantly better sample handling capacity on the ProSwift monolith. Based on these comparisons the ProSwift WAX monolith has at least 10 times the capacity of the nonporous anion-exchanger, on a bed volume basis.

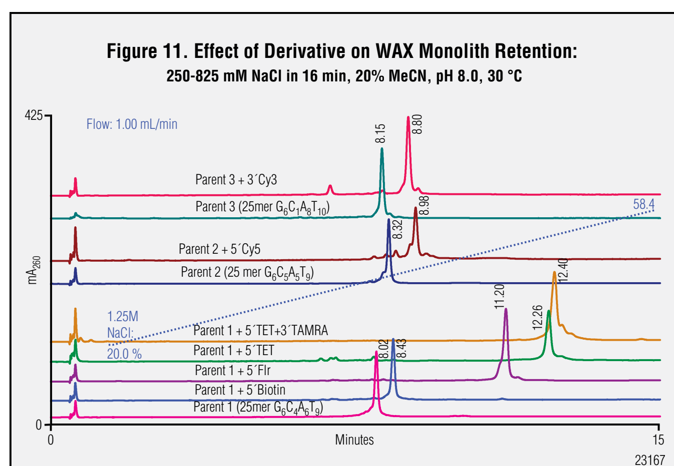


Figure 11. Effect of Derivative on WAX Monolith Retention:
250-825 mM NaCl in 16 min, 20% MeCN, pH 8.0, 30 °C

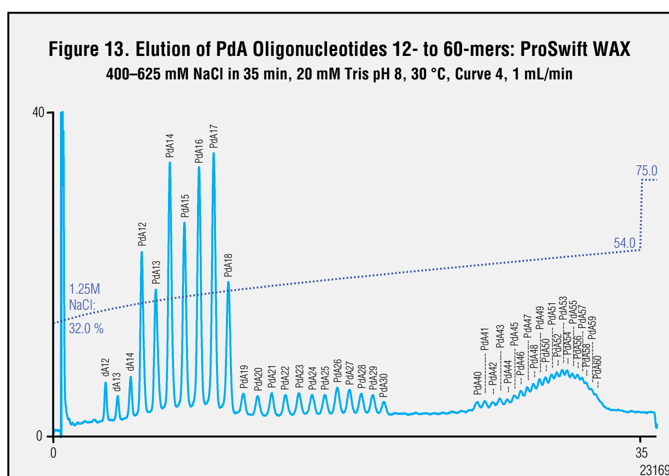


Figure 13. Elution of PdA Oligonucleotides 12- to 60-mers: ProSwift WAX
400–625 mM NaCl in 35 min, 20 mM Tris pH 8, 30 °C, Curve 4, 1 mL/min

Like the DNAPac PA200, the ProSwift WAX monolith resolves oligo-nucleotides harboring fluorescent derivatives from their underivatized parents. In Figure 11, the ProSwift WAX monolith resolves each of six different popular derivatives from their parent oligonucleotides at pH 8 and 30 °C.

The ProSwift WAX column fully resolves very similar oligonucleotides (in Figure 13 homopolymers of dA) up to 40 or more bases long, from their "n-1" failure sequences.

CONCLUSIONS

Observations on RNA with Aberrant 2´–5´ Linkages

- Phosphodiesterase-II does not appear to cleave at 2´– 5´ linkages.
- This Phosphodiesterase-II product appears to skip over 2´– 5´ linkages to produce partial digests from oligos harboring 5´-end located 2´– 5´ linkages.

DNAPac PA200

- Resolves rA 21-mer 2´– 5´ linkage isomers.
- Partially resolves 7 of 11 mixed-base 21-mer linkage isomers.
- Resolves PDAse-II degradation products of each of 11 mixed-base 2´– 5´ linkage isomers to unique positions.
- Is recommended for analytical application at low sample concentrations.

ProSwift WAX-1S Monolith

- ProSwift WAX monolith Exhibits >10-fold more capacity per bed volume, than the nonporous anion-exchanger.
- Resolves rA 21-mer 2´– 5´ linkage isomers, and partially resolves all eleven mixed-base 21-mer linkage isomers.
- Resolves PDAse-II degradation products of each of 11 mixed-base 2´– 5´ linkage isomers to unique positions.
- Can resolve oligonucleotides of 40 or more bases.
- Recommended for high resolution purification and analysis.
- Will be available in larger sizes with a linear increase in sample handling (up to ~5 mg on 10 × 100 mm format).

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